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Retinoic Acid Induces Neuronal Differentiation of a Cloned Human Embryonal Carcinoma Cell Line *in Vitro*

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The human embryonal carcinoma cell lines NT2/D1 and NT2/B9, clonally derived from Tera-2, differentiate extensively *in vitro* when exposed to retinoic acid. This differentiation is marked by the appearance of several morphologically distinct cell types and by changes in cell surface phenotype, particularly by the disappearance of stage-specific embryonic antigen-3 (SSEA-3), which is characteristically expressed by human EC cells. Among the differentiated cells are neurons, which form clusters interconnected by extended networks of axon bundles, and which express tetanus toxin receptors and neurofilament proteins. These observations constitute the first instance of extensive somatic differentiation of a clonal human EC cell line *in vitro*.

INTRODUCTION

Teratocarcinomas occur in both humans and mice (Stevens, 1967; Damjanov and Solter, 1974), but many studies point to marked differences in their developmental and biochemical properties (Andrews *et al.*, 1980, 1982; Andrews, 1982, 1983a; Avner *et al.*, 1981; Cotte *et al.*, 1982; Damjanov and Andrews, 1983; Damjanov *et al.*, 1982; Ilgren and Vaux, 1983; McIlhinney and Patel, 1983). Nevertheless, while the embryonal carcinoma (EC) stem cells of murine teratocarcinomas have been widely used as tools for the investigation of murine embryogenesis (e.g., for reviews see Brûlet *et al.*, 1983; Graham, 1977; Hogan *et al.*, 1983; Martin *et al.*, 1980; Solter and Damjanov, 1979), corresponding experimental investigation of cellular differentiation in human teratocarcinomas has been limited by the lack of pluripotent stem cell lines with the capacity for extensive cellular differentiation into somatic derivatives *in vitro*. Even though the EC cells of the human teratocarcinoma cell line Tera-2 (Fogh and Trempe, 1975; Andrews *et al.*, 1983a,b; 1984a) form well-differentiated tumors containing endodermal, mesodermal, and ectodermal derivatives when injected into athymic (*nu/nu*) mice, they show only limited spontaneous differentiation *in vitro*, comparable with that observed from other human EC cells (Andrews *et al.*,, 1980, 1982; Bronson *et al.*, 1983; Cotte *et al.*, 1982; McIlhinney *et al.*,, 1983; Zeuthen *et al.*, 1980). Retinoic acid, at concentrations as low as 10^{-7} to 10^{-9} *M*, induces the differentiation of many murine EC cell lines (Strickland and Mahdavi, 1978; Jetten *et al.*, 1979; Jones-Villeneuve *et al.*, 1982; Liesi *et al.*, 1983), but human teratocarcinoma cultures have so far proved unresponsive to this agent (Cotte *et al.*, 1982; Matthaei *et al.*, 1983). I now report that, in the presence of retinoic acid, clonally derived human EC cell lines (NT2/D1 and NT2/B9) (Andrews *et al.*, 1983a,b; 1984a) isolated from Tera-2 undergo differentiation *in vitro* into several cell types including neurons.

MATERIALS AND METHODS

Cell Culture and Retinoic Acid Treatment

The isolation and characteristics of the human EC cell lines NT2/B9 and NT2/D1, cloned from the teratocarcinoma line Tera-2 (Fogh and Trempe, 1975) have been previously described in detail (Andrews *et al.*, 1983a,b, 1984a). Stock cultures, predominantly exhibiting a human EC phenotype, were maintained in Dulbecco's modified minimal essential medium (high glucose formulation; Flow Laboratories) supplemented with 10% fetal calf serum (FCS) as before. Experimental cultures to be exposed to retinoic acid were established by seeding cells, harvested from the stock cultures by treatment with 0.25% (w/v) trypsin-2 m*M* EDTA, at a density of 10^6 cells per 75-cm^2 flask. All-*trans*-retinoic acid (Eastman Kodak), diluted from a 10^{-2} *M* stock solution in dimethyl sulfoxide (DMSO), was included in the medium of these cultures as required. For estimating plating efficiencies, between 100 and 200 cells were seeded per 25-cm^2 flask in the presence or absence of retinoic acid. After 2 weeks, the cultures were fixed with ethanol:acetic acid (9:1, v/v), and stained with Giemsa. The colonies were counted, and expressed as a fraction of the number of cells seeded.

Cell Surface Antigens

Cell surface antigen expression was analyzed by indirect immunofluorescence assayed by flow cytofluori-

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metry on an Ortho Cytofluorograf, as previously described (Andrews *et al.*, 1980, 1982). The following monoclonal antibodes were used: anti-SSEA-1 (Solter and Knowles, 1978); anti-SSEA-3 (Shevinsky *et al.*, 1982); W6/32 (anti-HLA-A, B, C; Barnstable *et al.*, 1978); TRA-2-49/6E (anti-liver alkaline phosphatase, Andrews *et al.*, 1984b); ALPp/Sp2/3 (anti-placental alkaline phosphatase; Gogolin *et al.*, 1982; Slaughter *et al.*, 1981); ALPp/Sp2/5 (reactive with both placental and intestinal alkaline phosphatases; Gogolin *et al.*, 1982; Slaughter *et al.*, 1981); and TRA-1-60, a monoclonal antibody which recognizes a cell surface antigenic determinant expressed by human EC cells but few other cell types (Andrews, 1983; and unpublished observations). All reagents were pretitrated to confirm the concentration required to give maximal binding and used as ascites diluted 1:100, with the exception of TRA-2-49/6E which was used as a 10-fold concentrated culture supernatant. Ascites from mice bearing the P3X63Ag8 myeloma (Kohler and Milstein, 1975) was used as a negative control. For flow cytofluorimetry, a fluorescence threshold was set so that approximately 95% of the cells which had been incubated with the P3X63Ag8 negative control antibody fluoresced with a lower intensity than this threshold; cells fluorescing with a greater intensity after reaction with the specific antibodies were counted as antigen positive. Fluorescein isothiocyanate (FITC)-conjugated rabbit IgG anti-mouse IgM (for anti-SSEA-3, anti-SSEA-1 and TRA-1-60) or FITC-conjugated rabbit IgG anti-mouse IgG, heavy- and light-chain specific (for W6/32, TRA-2-49/6E, ALPp/Sp2/3, ALPp/Sp2/5), purchased from Cappel Laboratories (Cochranville, Pa.), were diluted 1:10, and used as the detecting antibodies.

Neuronal Markers—Tetanus Toxin Receptor and Neurofilaments

These markers were detected by indirect immunofluorescence assays, *in situ*, on cultures grown on glass coverslips. The reactivity with tetanus toxin was assessed on unfixed cultures: coverslips were incubated sequentially for 30 min each, at room temperature, with 10 μg/ml tetanus toxin, rabbit anti-tetanus toxin serum (diluted 1:80, preabsorbed with NT2/D1 EC cells), and FITC-conjugated goat IgG anti-rabbit IgG (Cappel Laboratories; diluted 1:10). Dilutions were made and coverslips washed in Dulbecco's phosphate-buffered saline (PBS) containing 5% FCS. After the final wash, they were mounted in 50% glycerol in PBS. For a negative control, tetanus toxin was omitted from the first incubation. The presence of neurofilaments was detected on similar coverslips, first fixed for 10 min in cold acetone, and subsequently incubated with 7H11 (ascites, diluted 1:100), a murine monoclonal antibody with specificity for the 200,000-mol wt neurofilament protein (Drager *et al.*, 1984) followed by fluorescein-conjugated rabbit IgG anti-mouse IgG (Cappel Laboratories; diluted 1:10). After the final wash, these coverslips were mounted in UVinert (Gurrs). Other monoclonal antibodies to the 200,000-mol wt neurofilament polypeptide (Wood and Anderton, 1981) and to a determinant common to the 150,000- and 200,000-mol wt neurofilament polypeptides (Lee *et al.*, 1982) were also used in confirmatory experiments. For a negative control, the 7H11 antibody was replaced with P3X63Ag8 ascites. Also, reactivity with two monoclonal antibodies specific for glial fibrillary acidic protein (GFAP) (Lee *et al.*, 1984; Marsden *et al.*, 1983) was tested. Preparations were viewed both under UV epi-illumination and bright-field illumination.

Secreted Products

Culture supernatants were assayed for human chorionic gonadotropin (HCG) and alpha fetoprotein (AFP) by radioimmunoassay as previously described (Andrews *et al.*, 1980, 1984a). For comparison, HCG production by Bewo, a gestational choriocarcinoma cell line (Patillo and Gey, 1968), and AFP production by HepG2, a hepatoblastoma cell line (Aden *et al.*, 1979) were also assayed. Production of laminin was assayed by immunoprecipitation from culture supernatants of cells incubated with [^{35}S]methionine, and by immunofluorescence of acetone-fixed cell monolayers, as described previously (Andrews *et al.*, 1983b, 1984a). Rabbit anti-rat laminin sera, cross-reactive with human laminin, was kindly provided by Dr. Antonio Martinez-Hernandez (Hahnemann University Medical School).

RESULTS

When NT2/D1 or NT2/B9 cells were exposed to 10^{-5} *M* retinoic acid for 7 days, the morphology of the cells was markedly different from that in control cultures grown in the absence of retinoic acid (Fig. 1a,b). Subsequently, continued growth of the retinoic acid-treated cultures resulted in the appearance of islands of cells exhibiting various morphologies and, in particular, the appearance of cells that resembled neurons (Fig. 1c).

In parallel with the disappearance of EC cell morphology, the expression of SSEA-3, a cell surface antigen characteristically expressed by human EC cells (Andrews *et al.*, 1982; Damjanov *et al.*, 1982; Shevinsky *et al.*, 1982), was extinguished (Fig. 2a) following exposure to 10^{-5} and 10^{-6} *M* retinoic acid; at 10^{-7} *M* retinoic acid, many cells continued to express SSEA-3 and also retained an EC-like morphology. Retinoic acid at these concentrations was not toxic since the cultures in the presence of retinoic acid grew well, although more slowly than the control cultures (Fig. 2b). Also, the plating efficiency was similar (Fig. 2c). The apparent small re-

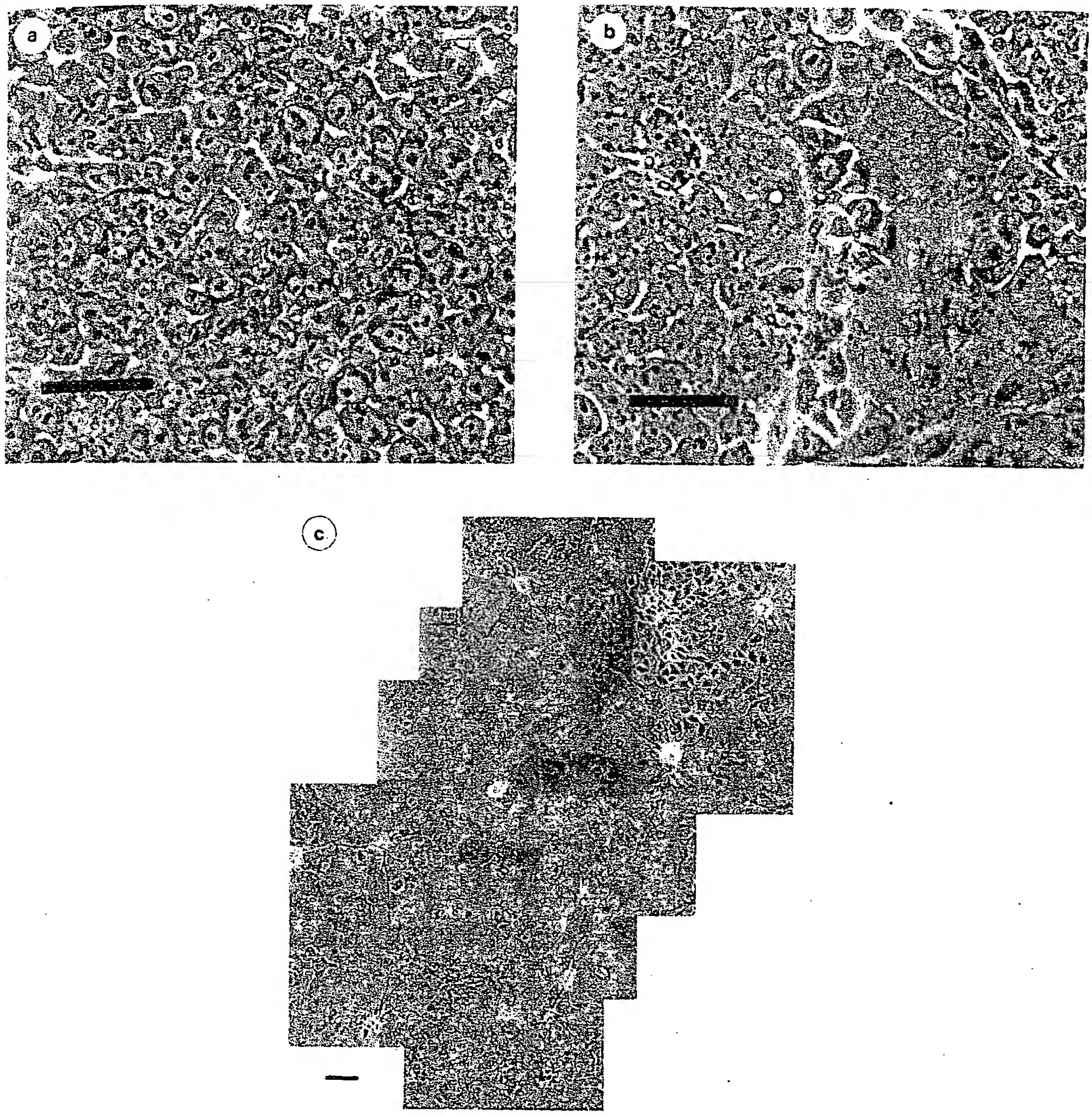

FIG. 1. The morphology of cells differentiating from NT2/D1 human EC cells in response to retinoic acid. a and b, NT2/D1 cells are shown 7 days after seeding at 10^6 cells per 75-cm^2 flask in the absence of retinoic acid (EC morphology) and in the presence of 10^{-5} *M* retinoic acid, respectively. Bar = 50 μm. c, Differentiated cells in an NT2/D1 culture first exposed to 10^{-5} *M* retinoic acid for 3 weeks, after which they were harvested and reseeded at 5×10^6 cells per 75-cm^2 flask (split ratio, approx 1:4) and maintained for a further 4 weeks in the absence of retinoic acid. Note the various morphologies of the differentiated cells, especially the neuronal-like cells which form an extended network over the surface of the culture. Bar = 100 μm.

duction in plating efficiency in the presence of retinoic acid compared to the control cultures probably rests on the different growth rate and morphology of colonies composed of either differentiated or EC cells: the differentiated cells grew as monolayers forming small, lightly staining colonies, whereas the EC cells grew in multicellular layers which stained darkly and were more readily detected. Most colonies in all three concentra-

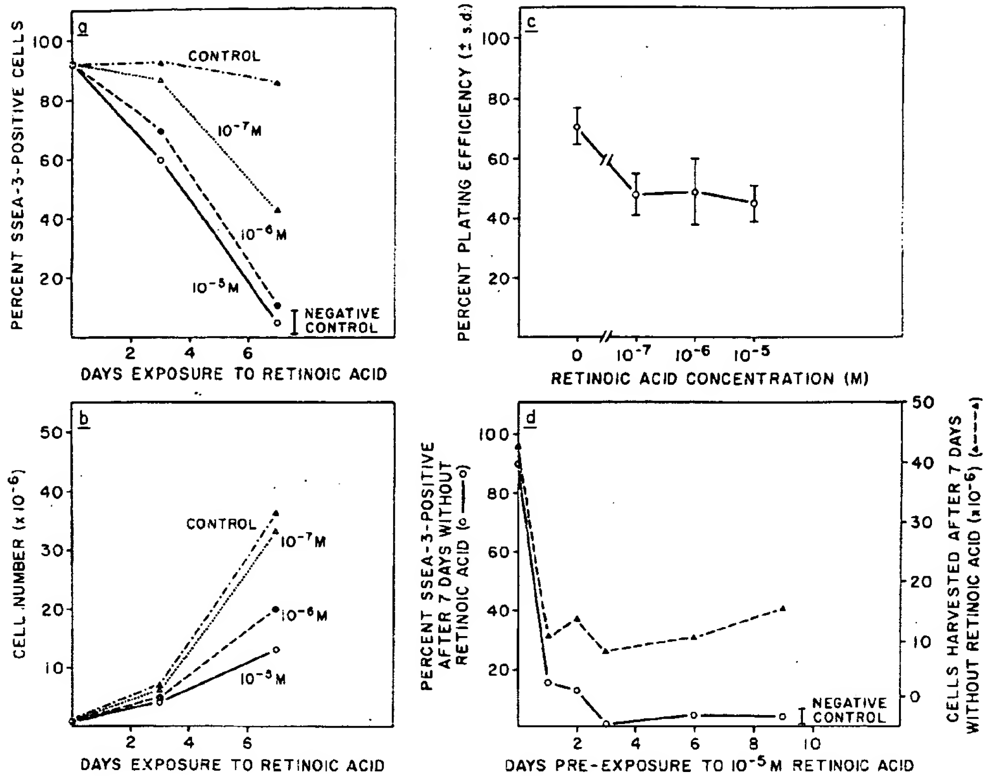

FIG. 2. The effect of retinoic acid on SSEA-3 expression and growth of NT2/D1 human EC cells. a and b, Time course and concentration dependence of the action of retinoic acid on SSEA-3 expression and growth rate, respectively. The cells were seeded at 10^6 per 75-cm^2 flask with 10^{-5} *M* (O), 10^{-6} *M* (●), 10^{-7} *M* (△), or no (▲) retinoic acid. SSEA-3 expression was assayed by flow cytofluorimetry; the proportion of cells reactive with P3X63Ag8 negative control antibody is indicated by the bar in Figs. 1a and d. c, Plating efficiency of NT2/D1 cells in the presence of retinoic acid. The means and standard deviations of quadruplicate determinations are shown. d, The stability of the retinoic acid-induced phenotype. Cultures of NT2/D1 cells, seeded at 10^6 per 75-cm^2 flask, were grown in the presence of 10^{-5} *M* retinoic acid for between 1 and 9 days, as shown on the abscissa. The cells were then trypsinized and reseeded at 10^6 cells per 75-cm^2 in the absence of retinoic acid. After a further 7 days the cells were counted and their expression of SSEA-3 analyzed (ordinates). Note that the cells exposed to retinoic acid for any of the time periods subsequently grew, but more slowly (three to four doubling divisions in 7 days) than control cells not exposed to retinoic acid (five to six doubling divisions in 7 days). Also the expression of SSEA-3 was markedly reduced after a 1-day preexposure to retinoic acid, and not subsequently detectable after a 3-day preexposure.

tions of retinoic acid used were of the differentiated type, although 3% of the colonies in 10^{-7} *M* retinoic acid, compared to about 0.14% in the presence of 10^{-5} and 10^{-6} *M* retinoic acid, resembled the darkly staining EC colonies of the control cultures.

The differentiated phenotype induced by retinoic acid in NT2/D1 or NT2/B9 cells was stable. When the cells were grown in 10^{-5} *M* retinoic acid for 1 day and replated in its absence, most of the cells underwent morphological differentiation and less than 20% of the cells were SSEA-3-positive 7 days later; preexposure to retinoic acid from between 3 and 7 days was sufficient to induce differentiation of almost all the SSEA-3-positive EC cells (Fig. 2d). In subsequent experiments to study long-term changes, cultures treated with retinoic acid for 3 weeks were maintained for up to 6 months in its absence. During this time only a few isolated colonies of EC cells developed, presumably from residual stem cells. Provided the cultures were not trypsinised and reseeded, these colonies remained discrete and did not overgrow the differentiated cells. Rather, they budded off into the culture supernatant as cell clusters which did not reattach well to the layer of differentiated cells. However, when transferred to a new flask, they did reattach and formed homogeneous cultures of EC-like cells, expressing SSEA-3. These cells were not resistant to further treatment with retinoic acid.

Control cultures incubated with 0.1% DMSO, the final concentration in cultures treated with 10^{-5} *M* retinoic acid, resembled untreated cultures in their morphology, growth rate and expression of SSEA-3. Further, retinoic acid diluted from a stock solution in ethanol was as

effective in inducing differentiation as retinoic acid diluted from a stock solution in DMSO (data not shown), thus eliminating the possibility that the observed differentiation was due either to DMSO alone or to a synergistic action of DMSO and retinoic acid.

In addition to the loss of expression of SSEA-3, and the changes in growth characteristics and morphology, the expression of several other cell surface antigens also changed following exposure to retinoic acid (Fig. 3). Thus the antigen detected by TRA-1-60 was extinguished (Fig. 3b). Although its molecular nature has not yet been determined in detail, this antigen is associated with polypeptides of 200,000 and 400,000 mol wt (Andrews, 1983; unpublished observations), and is apparently distinct from the multiple glycoproteins and glycolipids carrying the globoseries oligosaccharides reactive with anti-SSEA-3 (Shevinsky *et al.*, 1982; Kannagi *et al.*, 1983). The TRA-1-60-reactive polypeptides are not laminin, which is not synthesized by NT2/D1 or NT2/B9 human EC cells (Andrews *et al.*, 1984a). SSEA-1, a cell surface carbohydrate epitope which commonly appears upon the

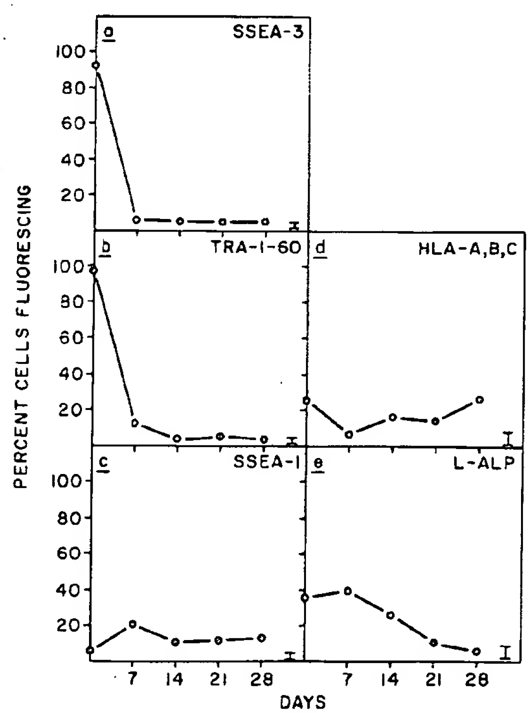

FIG. 3. Changes in surface antigen expression by NT2/D1 cells exposed to retinoic acid. The proportion of cells reactive with different antibodies was assayed by flow cytofluorimetry of cultures continuously exposed to 10^{-5} *M* retinoic acid for the time periods indicated on the abscissas: a, Anti-SSEA-3; b, TRA-1-60; c, anti-SSEA-1; d, W6/32, anti-HLA-A,B,C; e, TRA-2-49/6E, anti-L-ALP. The bar in each figure indicates the proportion of cells reactive with P3X63Ag8 negative control antibody.

TABLE 1
LACK OF HCG OR AFP PRODUCTION BY NT2/D1 DIFFERENTIATED CULTURES

	Time in retinoic acid	HCG ($ng/10^6$ cells/day)	AFP ($ng/10^6$ cells/day)
Experiment 1	7 days	<0.07	<0.4
Experiment 2	21 days	<0.08	<0.6
Controls			
NT2/D1 (no retinoic acid)		<0.07	<0.4
Bewo (gestational choriocarcinoma)		163	<0.8
HepG2 (hepatoblastoma)		nd	595

Note. Cultures of NT2/D1 were allowed to differentiate in the presence of 10^{-5} *M* retinoic acid for the periods shown. Culture supernatants, in contact with the cells for 7 days during exposure to retinoic acid (Experiment 1) or for 7 days following exposure to retinoic acid (Experiment 2) were concentrated 10-fold, and assayed for HCG and AFP. The limiting values for production of these proteins was calculated based upon the number of cells at the time of harvest and assay sensitivities of 0.6 ng HCG/ml and 2.2 ng AFP/ml.

limited differentiation induced by low density culture of human EC cells (Andrews *et al.*, 1982, 1984a) appeared transiently as if on a population of cells which disappears with further retinoic acid treatment (Fig. 3c). HLA-A,B,C expression was generally reduced soon after retinoic acid was added to the culture medium, but the proportion of cells expressing this antigen subsequently rose in longer term cultures (Fig. 3d). By contrast, expression of an antigen associated with liver alkaline phosphatase (L-ALP) usually remained at a level similar to that of control cultures for a period of up to two weeks, but subsequently fell to low levels (Fig. 3e). Expression of antigens specifically associated with the placental and intestinal isozymes of ALP was not detected in either the control or differentiated cultures (data not shown).

To establish whether any of the morphologically diverse cells that appeared in response to retinoic acid treatment corresponded to extraembryonic tissues such as the trophoblast or yolk sac, the biosynthesis of proteins characteristic of these cell types was assayed, but no synthesis of either HCG or AFP (Table 1) or of laminin (data not shown) was found. Particularly striking, however, were the small neuron-like cells shown in Fig. 1c. These formed clusters over the other differentiated cells and, after several weeks, extended bundles of axon-like processes which interconnected distant clusters. Although exposure to retinoic acid for 7 days was sufficient to induce their eventual appearance, the treated cells had to be cultured for a further 7 to 14 days before these neuron-like cells became evident. To confirm their neuronal character, the expression of a ganglioside cell surface receptor for tetanus toxin (van Heyningen, 1963) and the presence of neurofilaments, both characteristic

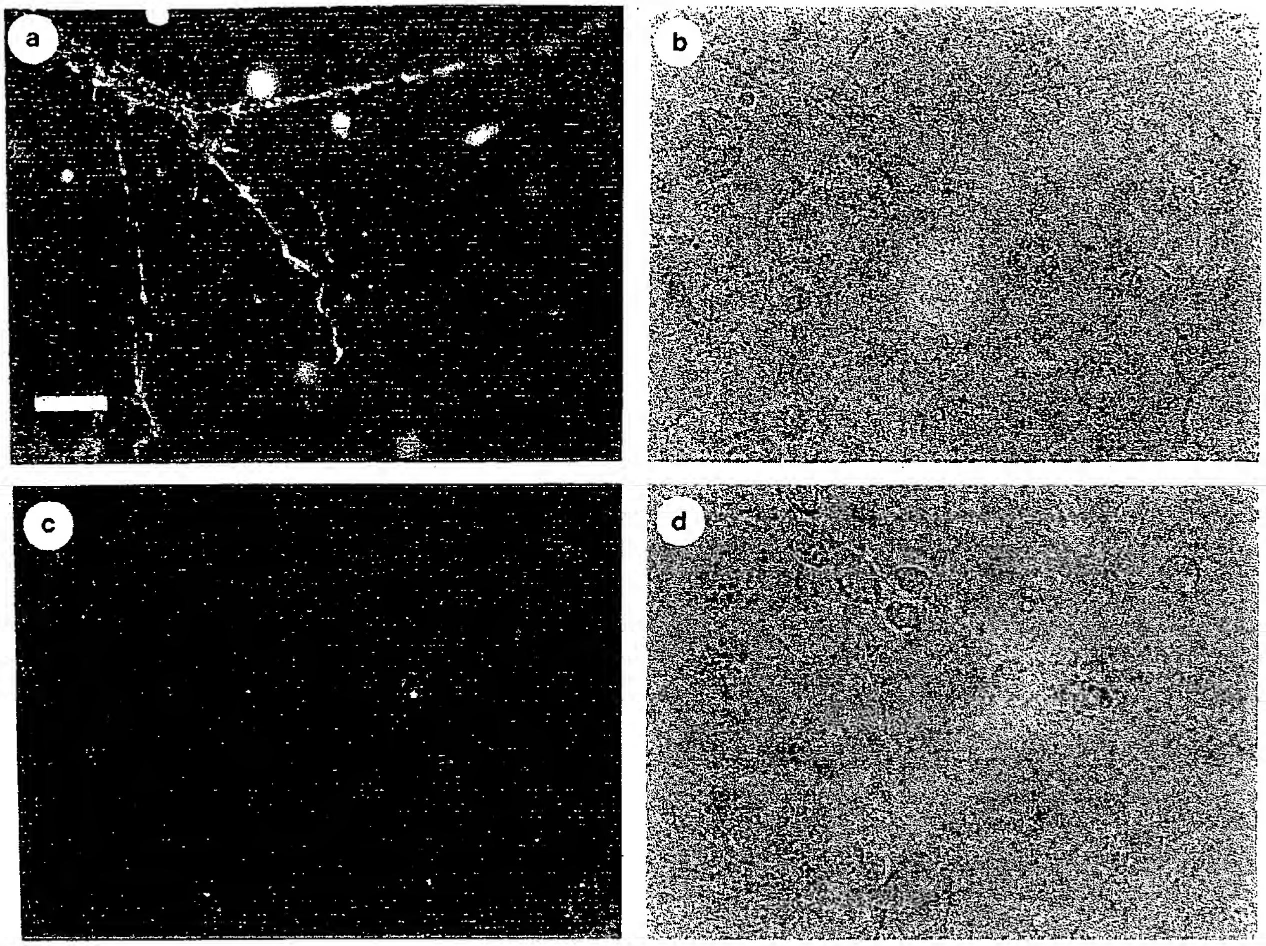

FIG. 4. Binding of tetanus toxin and monoclonal anti-neurofilament antibody to NT2/D1-derived neuron-like cells. These cultures were obtained by preexposing NT2/D1 EC cells to 10^{-5} *M* retinoic acid for 3 weeks, followed by growth on glass coverslips for 4 weeks in the absence of retinoic acid. a and b, Tetanus toxin binding; c and d, negative control, tetanus toxin omitted; e and f, binding of 7H11 to monoclonal antibody neurofilaments; g and h, negative control, binding of antibody from the P3X63Ag8 myeloma. a, c, e, and g were viewed by UV epi-illumination and b, d, f, and h by bright-field illumination. The bar represents both 20 (a-d) and 100 μm (e-h).

markers of neurons (Mirsky *et al.*, 1978; Fields *et al.*, 1978; Lazarides, 1980; Osborn and Weber, 1983) were examined. Indeed the neuron-like cells did react with tetanus toxin and with the 7H11 monoclonal antibody specific for the 200,000-mol wt neurofilament protein (Drager *et al.*, 1984) (Fig. 4). Similar reactivity to that of 7H11 was observed when two other monoclonal antibodies to neurofilament polypeptides were tested (not shown). Neither the EC stem cells nor the other differentiated cells reacted with these reagents. Because a recent report noted reactivity of tetanus toxin with a subclass of glial cells (Raff *et al.*, 1983), the possible presence of the glial cell intermediate filament protein, GFAP, was also examined but no reactivity with two monoclonal antibodies specific for GFAP was found (not shown).

DISCUSSION

These results constitute the first example of extensive differentiation of clonal human EC cells into a range of somatic cell types *in vitro*. The data presented here were obtained from experiments with the NT2/D1 clone, but NT2/B9 cells were also found to differentiate in a similar way. Differentiation was marked by the appearance of several cell types including neurons, and by changes in the expression of several surface antigens, including the disappearance of SSEA-3, the TRA-1-60 antigen, and liver alkaline phosphatase. This retinoic acid-induced differentiation of human EC cells *in vitro* is evidently a complex process, since the heterogeneity of the cultures increased with time after the addition of retinoic acid, and recognizable neuronal cells appeared

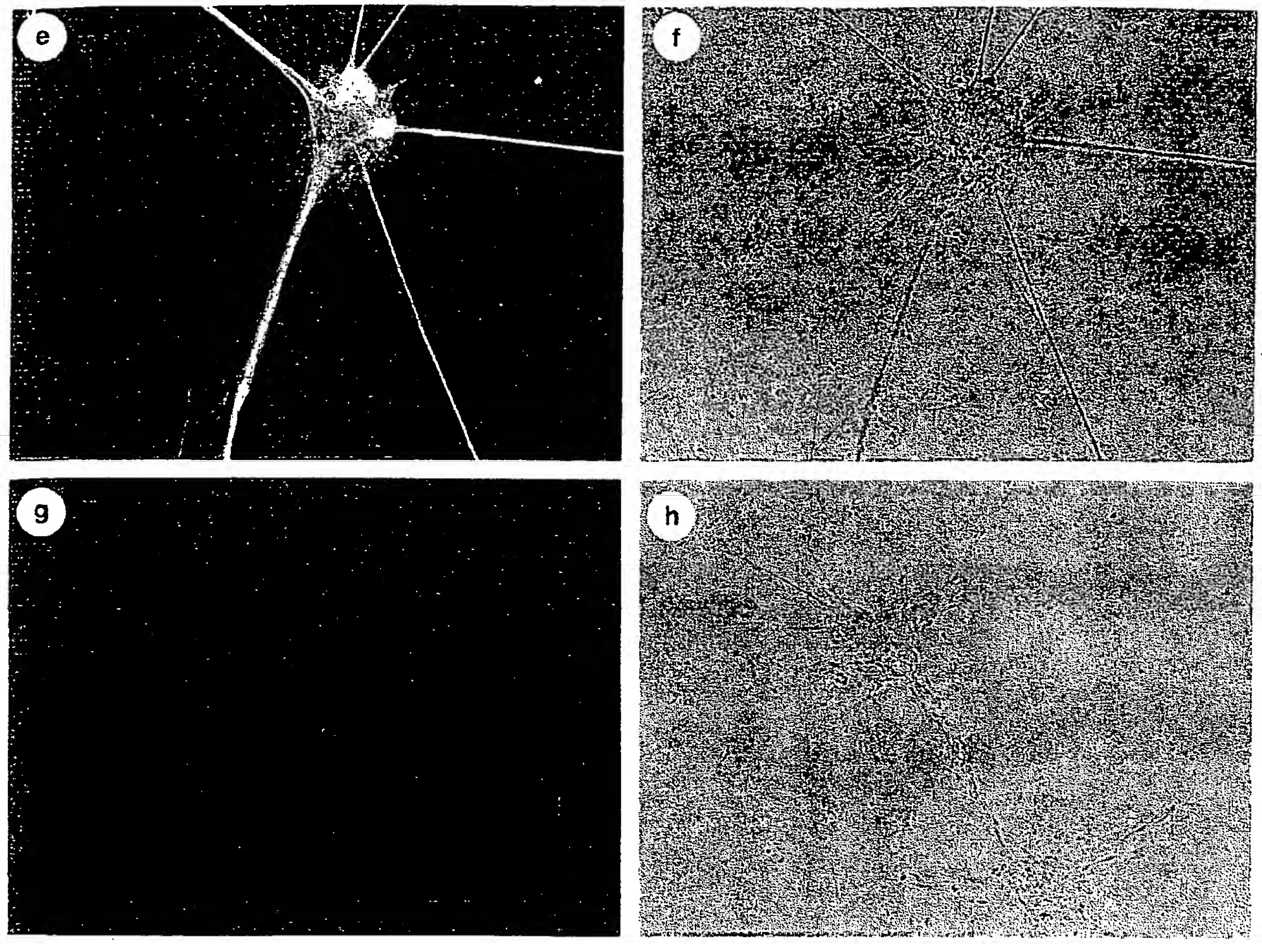

FIG. 4—*Continued.*

only after other changes had already occurred. These observations suggest multiple pathways of differentiation, and raise the possibility that interactions with other derivatives of the EC stem cells may be required to induce further differentiation of intermediate neuronal precursor cells into mature neurons. The identity of the other differentiated cells is not yet known. However, differentiation along trophoblastic or yolk sac lineages seems unlikely since the synthesis of chorionic gonadotropin, alpha fetoprotein, or laminin was not detected. The lack of GFAP also suggests that significant glial cell differentiation does not occur.

By comparison with murine EC cells, a relatively high concentration of retinoic acid was required to induce the differentiation of Tera-2-derived human EC cells. Further, the time course of the appearance of neuronal cells was longer than that reported for murine EC cell lines (Jones-Villeneuve *et al.*, 1982). However, it should be noted that the response of different murine EC cell lines to retinoic acid is very variable (e.g., Strickland and Madhavi, 1978; Jetten *et al.*, 1979; Jones-Villeneuve *et al.*, 1982).

It is unknown why Tera-2-derived EC cells should respond to retinoic acid whereas other human EC cell lines do not (Cotte *et al.*, 1982; Matthaei *et al.*, 1983). Preliminary studies have also suggested that hexamethylene bisacetamide may also induce morphological changes, although not neuronal differentiation, in cultures of NTera-2 cells, but not of other human EC cells (unpublished observations). Other agents, such as retinoic acid analogues remain to be examined. Of the clonal human teratocarcinoma cell lines so far studied only the Tera-2-derived EC cells have shown evidence of extensive differentiation into somatic derivatives in xenograft tumors (Andrews *et al.*, 1983a,b, 1984a). However, the spontaneous differentiation of these, and other human EC cells, *in vitro* is much more limited (Andrews *et al.*, 1982; 1983a,b, 1984a) and clearly different from

that induced by retinoic acid; for example, neuronal cells have not been found. Also, one of the chief characteristics of the spontaneous differentiation is the appearance of cells expressing the surface antigen SSEA-1, whereas such cells appeared only transiently when the Tera-2 cloned lines were treated with retinoic acid. That Tera-2 EC cells should differentiate extensively when grown as a xenograft but not spontaneously *in vitro* suggests that either retinoids provided by the mouse host, or other agents whose action is mimicked by retinoic acid, might promote differentiation *in vivo*. Nevertheless, differentiation in the xenografts was not identical to that induced by retinoic acid *in vitro;* neuronal elements were detected in both instances, but whereas gland-like structures expressing the intestinal isozyme of ALP were found in xenograft tumors (Andrews *et al.*, 1984a), no cells expressing this isozyme were detected in the retinoic acid-induced differentiated cultures.

Irrespective of the mechanism of action of retinoic acid on these human EC cells, this system now provides a tool for the study of human cellular differentiation *in vitro* in a way which may be pertinent to human embryonic cellular differentiation. Further, the extensive neuronal interconnections observed suggest that this model system might also be useful for the study of neuronal differentiation and the formation of neural networks in the human embryo.

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